

Anti-inflammatory therapy and cardiovascular disease in inflammatory arthropathies

Effects of TNF- α antagonists on vascular function and structure

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Common abbreviations

ADMA	Asymmetric Dimethylarginine
AIx	Augmentation Index
aPWV	Aortic Pulse Wave Velocity
AS	Ankylosing Spondylitis
CCP	Cyclic Citrullinated Peptide
CIMT	Carotid Intima Media Thickness
COX-2	Cyclooxygenase-2
CRP	C-reactive Protein
CVD	Cardiovascular Disease
DAS28	Disease Activity Score (based on 28 joints)
DDAH	Dimethylarginine Dimethylaminohydrolase
ESR	Erythrocyte Sedimentation Rate
FMD	Flow Mediated Dilatation
HAQ	Health Assessment Questionnaire
LDL	Low Density Lipoprotein
MAP	Mean Arterial Pressure
MMP	Matrix Metalloproteinase
MRP	Myeloid Related Protein
MTX	Methotrexate
NO	Nitric Oxide
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
NF- κ B	Nuclear Factor- κ Beta
PsA	Psoriatic Arthritis
RA	Rheumatoid Arthritis
RAGE	Receptor for Advanced Glycation End products
RCT	Randomized Controlled Trial
SD	Standard Deviation
TLR	Toll-Like Receptor
TNF- α	Tumor Necrosis Factor- α
TNFR	Tumor Necrosis Factor Receptor
VAS	Visual Analogue Scale

List of papers

1. Angel K, Provan SA, Hammer HB, Mowinckel P, Kvien TK, Atar D. Changes in arterial stiffness during continued infliximab treatment in patients with inflammatory arthropathies. *Fundamental & Clinical Pharmacology* 2011;25:511-517.
2. Angel K, Provan SA, Gulseth HL, Mowinckel P, Kvien TK, Atar D. Tumor Necrosis Factor- α antagonists improve aortic stiffness in patients with inflammatory arthropathies: A controlled study. *Hypertension* 2010;55:333-338.
3. Angel K, Provan SA, Fagerhol MK, Mowinckel P, Kvien TK, Atar D. Effect of 1-year anti-TNF- α therapy on aortic stiffness, carotid atherosclerosis and calprotectin in inflammatory arthropathies: A controlled study. *American Journal of Hypertension*, advance online publication 1 March 2012; doi: 10.1038/ajh.2012.12.
4. Angel K, Provan SA, Mowinckel P, Seljeflot I, Kvien TK, Atar D. The L-arginine/asymmetric dimethylarginine ratio is improved by anti-Tumor Necrosis Factor- α therapy in inflammatory arthropathies. Associations with aortic stiffness. *Submitted*.

1 Introduction and background

Rheumatoid arthritis (RA), ankylosing spondylitis (AS) and psoriatic arthritis (PsA) are the three most prevalent chronic inflammatory arthropathies. Cardiac involvement such as valve disorders, peri-/myocarditis and conduction defects have been recognized for several years, particularly in AS and RA. However, in recent years, several studies have indicated that inflammatory arthropathies also are associated with an excess risk of cardiovascular disease (CVD) such as coronary heart disease caused by atherosclerosis. Atherosclerosis, which is fatty deposits in the artery walls, leads to structural changes of the vessels, and is often preceded or coincidental with functional changes of the arteries. During the last decades, atherosclerosis has emerged as an inflammatory disease (1), and the inflammatory processes in atherosclerosis and inflammatory arthropathies share several features (2). Drugs targeting the pro-inflammatory cytokine Tumor Necrosis Factor- α (TNF- α) have since their introduction in the late 90's revolutionized the treatment of inflammatory arthropathies. Against this background, the aim of this thesis was to explore whether anti-TNF- α therapy could be beneficial with regard to vascular pathology in inflammatory arthropathies. In 2005 when this thesis was initiated, knowledge about vascular effects of anti-TNF- α therapy was limited; it will be described in the *Introduction and background* part of this dissertation. Since then, the research activity within this field has increased considerably, and the results from the studies in this thesis will be discussed in light of more recent publications in the *General discussion* part.

1.1 Inflammatory arthropathies

The prevalence of RA is about 0.5-1.0 % in Caucasians, but varies across populations (3). Women are affected two to five times more often than men. RA can occur in patients at any age, but the highest incident rates are at the ages between 60 and 70 (4). The disease is characterized by synovial inflammation that commonly affects small joints of the hands and feet symmetrically, but also frequently involve larger joints like the shoulder and knee. While the etiology of RA is largely unknown, it is often associated with anti-cyclic citrullinated peptide (anti-CCP) antibodies. Both genetic and environmental factor have been recognized to increase the risk of developing anti-CCP antibodies (5). Although disease activity may vary over time, RA is considered a progressive disease. If the disease is uncontrolled, it may lead to destruction of joints due to cartilage degradation and bone erosion.

AS and PsA belong to a group of interrelated inflammatory arthropathies which collectively are called spondyloarthropathies (6), or more recently spondyloarthritis (7).

Spondylarthropathies are not associated with anti-CCP antibodies, but show relationship with positivity of human leukocyte antigen-B27 (8). AS typically affects the sacroiliac joints, the axial skeleton and entheses, and is characterized by progressive spinal ankylosis due to new bone formation. AS is frequently associated with iridocyclitis, uveitis and inflammatory bowel disease. The disease characteristically affects young adults with a peak age of onset between 20 and 30 years. The prevalence of AS is lower than of RA, and AS is two to three times more frequent in men than in women. PsA affects women and men equally, with a prevalence of about 0.1 to 0.2 % (9). Disease onset is most frequent in the fifth decade of life. PsA is associated with psoriasis and typically affects peripheral joints such as the distal interphalangeal joints, but may also involve the spine. PsA commonly presents as an asymmetrical oligoarthritis or as a polyarthritis, although the pattern of arthritis may change over time. PsA with peripheral joint involvement is an erosive disease leading to resorption of cortical bone but is also associated with calcification and ossification at the sites of entheses (10).

1.2 Cardiovascular risk in RA

A considerable body of evidence supports the fact that RA is associated with an increased risk of cardiovascular mortality and morbidity compared to the general population. Van Doornum et al reviewed the results published prior to and including 2000 and showed that patients with RA had a reduced life expectancy by 3-18 years and a standardized mortality ratio of 1.7 (11). The excess mortality was mainly due to increased levels of cardiovascular death. Later, several other studies have reported similar findings (12-18).

Endothelial dysfunction (chapter 1.4.1) and arterial stiffening (chapter 3.2.1) are markers of vascular dysfunction and independent predictors of CVD in the general population (19;20). Compatible with the observed excess cardiovascular risk, also unfavorable changes in these vascular parameters have been demonstrated in patients with RA compared to control populations (21-27). Indicators of premature atherosclerosis such as increased levels of carotid intima media thickness (CIMT [chapter 3.2.2]) and carotid plaques have also been associated with RA (28-33).

Some cardiovascular risk factors, in particular smoking, dyslipidemia (increased atherogenic index; total cholesterol / high density lipoprotein cholesterol and increased levels of small dense low density lipoprotein cholesterol [LDL] particles) and insulin resistance, seem to be more frequent in patients with RA than in the general population (11;34;35). Furthermore, reduced physical activity due to the joint disease and fatigue might negatively influence the cardiovascular risk. Thus, an unfavourable cardiovascular risk profile might contribute to the accelerated atherosclerosis in RA. Nevertheless, the excess risk is only minimally attenuated by traditional risk factor adjustment (12;36), and also compared with patients with osteoarthritis, RA patients remain at higher cardiovascular risk (15;37). Several of the manifestations of RA such as higher deformed joint count, longer disease duration and higher disease activity have been associated with increased cardiovascular risk in different studies (11;14;36). These results indicate that the level of inflammation in patients with RA accelerates the atherosclerotic process, possibly through both an unfavourable influence on traditional risk factors and direct effects on the artery wall (11;34). In line with this interpretation, previous cross sectional studies have indicated that inflammatory markers correlate with vascular pathology both in patients with RA and in the general population (21;23;26;32;38-40).

1.3 Cardiovascular risk in AS and PsA

Patients with AS have already in the late 70's been reported to have increased all-cause mortality and augmented risk of cardiovascular death (41), and later similar findings have been reported in patients with PsA (42). However, available results on cardiac morbidity and mortality in these patients are limited and precise information about the specific type of cardiac disease has not been reported (41;43;44). In AS, cardiac involvement such as aortic root disease and valve disease have been associated with cardiovascular morbidity (45), and thus the cardiovascular risk due to atherosclerotic processes in these patients may be difficult to estimate. Nevertheless, Peters et al, in their review from 2004, concluded that available data all-together point to an increased risk of atherosclerotic CVD also in patients with AS and PsA (46). In concordance with this conclusion, Divecha et al have reported that patients with AS have increased levels of cardiovascular risk factors such as increased pulse pressure, fibrinogen levels and von Willebrand factor compared to controls (47). Furthermore, in the study by Divecha et al, systemic inflammation seemed to be associated with an unbeneficial

cardiovascular risk profile, which may indicate a role of systemic inflammation in premature atherosclerosis in AS similar to that in RA (47).

Previous data regarding vascular function and CIMT in PsA are missing, and only one study has assessed this issue in patients with AS. In this study, patients with AS had impaired endothelial function compared to healthy controls, whereas the CIMT values were not different between the two groups (48). However, this study included young patients at an average age of 37 years. Normally, atherosclerotic changes appear with age. Thus, studies with older patients or larger patient groups may be necessary to determine a possible presence of premature atherosclerosis in patients with AS and PsA.

1.4 Cardiovascular disease and inflammation

An increasing body of evidence supports the involvement of inflammatory mechanisms in atherosclerosis and vascular pathology, not only in patients with inflammatory arthropathies, but also in the general population (49). Cohort studies have shown that high sensitivity C-reactive protein (CRP) predicts incident myocardial infarction and cardiovascular death also after adjustment for traditional cardiovascular risk factors (50). In line with these results, inflammatory mediators are abundantly expressed during atherogenesis and are demonstrated to augment the atherosclerotic process (49). Patients with inflammatory arthropathies do not only express CRP in the high sensitivity range, but often have a high-grade systemic inflammation, which may accelerate the development of vascular pathology (34).

1.4.1 Inflammation and vascular function

The normal healthy endothelium is a major regulator of vascular homeostasis and modulates vascular tone and structure, smooth muscle cell proliferation and migration, coagulation, cell adhesiveness and vessel wall inflammation (49;51). Endothelial dysfunction, which refers to a disruption of the endothelium's vasoprotective properties, is considered as an initial step in the atherosclerotic process. Nitric oxide (NO), which is produced by the endothelial cells, mediates many of the endothelium's functions. One of the main characteristics of endothelial dysfunction is impaired endothelium dependent vasodilatation because of reduced NO bioavailability. Inflammatory processes have been demonstrated to suppress NO mediated vasodilatation partly through increased oxidative stress (52). Oxidative stress uncouples the enzyme nitric oxide synthase (NOS) which synthesizes NO from the amino acid L-arginine.

Another characteristic of endothelial dysfunction is endothelial activation. Activated endothelial cells express adhesion molecules such as vascular cell adhesion molecule-1, intercellular cell adhesion molecule-1 and e-selectin on their surface (49). Leucocytes are recruited to the artery intima by adhesion to the activated endothelium and migration through the endothelial layer. The migration process is facilitated by chemo attractant stimuli including chemokines (i.e monocyte chemoattractant protein-1) (53). Endothelial activation typically takes place at atherosclerosis prone sites such as arterial branching points and curvatures. Blood flow at these sites is characterized by turbulent flow and low average shear stress (tangential stress from the blood flow on the endothelium and the inner layers of the artery wall). Low shear stress has been demonstrated to promote expression of adhesion molecules, up-regulation of inflammatory genes and to reduce NO production in endothelial cells (54). Additionally, numerous stimuli including hypercholesterolemia and infiltration of LDL to the intima (55), hypertension, smoking and inflammatory mediators may induce endothelial activation (49).

The main determinants of large artery stiffness are the structural elements within the arterial wall, vascular muscle tone and blood pressure (20;56). Inflammatory mediated endothelial dysfunction may consequently promote functional arterial stiffening by reducing vascular vasodilatory capacity. Furthermore, the inflammatory cells recruited to the artery wall may directly affect smooth muscle cells, or contribute to changes in the composition of the artery wall and thus promote structural arterial stiffening (57). Inflammatory mediators including TNF- α stimulate release of matrix metalloproteinases (MMPs) (58). MMPs are a family of enzymes with proteolytic activity against extracellular matrix components such as elastin, collagen and proteoglycans. MMPs play an important role in vascular remodeling, and contribute to vascular adaptation and repair. However, with excessive MMP activity, inappropriate vascular remodeling may occur, and it has been shown that circulating MMP-9 levels are independently associated with aortic stiffness (59). Furthermore, inflammation may promote arterial stiffening through calcification of the artery wall. In vitro studies have demonstrated that monocyte- and macrophage derived TNF- α may induce alkaline phosphatase production, which is a marker of osteoblastic differentiation, and calcification in vascular smooth muscle cells (60;61). On the other hand, arterial stiffening may itself promote endothelial dysfunction by reducing shear stress and consequently endothelial NO production (62). Stiffer arteries may also directly advance atherosclerosis via increased pulse pressure

and changes in mechanical stress in the artery wall leading to damaged wall structure and thus increased susceptibility to atherosclerosis (20).

Infiltration of leucocytes and LDL might lead to structural atherosclerotic changes in the artery wall (chapter 1.4.2). However, alterations in blood flow, blood pressure and shear stress may also induce compensatory and non-atherosclerotic arterial wall thickening (63). Shear stress and tensile stress (the stretching force perpendicular to a longitudinal section through the artery wall or tangential to the wall) are major determinants of adaptive remodeling of the arterial wall. Both shear stress and tensile stress are kept relatively constant throughout the artery, and changes in shear stress and subsequently in lumen diameter are followed by changes in artery wall thickness to keep tensile stress constant (64).

1.4.2 Inflammation and atherosclerosis

After recruited to the artery wall (chapter 1.4.1), the leucocytes undergoes differentiation and activation. Under the influence of macrophage colony stimulating factor produced by endothelial cells and smooth muscle cells, monocytes differentiate into macrophages (65). Macrophage differentiation involves increased expression of pattern recognition receptors such as scavenger receptors and toll-like receptors (TLRs). Scavenger receptors mediate the uptake of numerous molecules and particles including oxidized LDL (66). Excessive uptake of oxidized LDL leads to accumulation of cytosolic cholesterol droplets, which induce macrophage foam cell formation. Ligand binding to TLRs triggers signaling that leads to activation of the intra nuclear factor- κ beta (NF- κ B). NF- κ B induces expression of pro-inflammatory cytokines that activate macrophages and other inflammatory cells. T-cells differentiate into T helper cells-1 and T helper cells -2 and regulatory T-cells after antigen presentation by macrophages or dendritic cells and co-stimulatory signaling. Previous results have indicated that T helper cells-1 dominates in atherosclerosis (67). T helper cells-1 responses generally amplify the inflammatory cascade by secretion of cytokines including interferon- γ and TNF- α . Also other inflammatory cell such as mast cells populate atherosclerotic lesions (68).

With time, lipoprotein infiltration and inflammation lead to development of atherosclerotic plaques. Atherosclerotic plaques contain inflammatory cells, as well as vascular endothelial cells, extracellular matrix, lipids and lipid-rich debris (69). In the centre of a plaque, macrophage foam cells and extracellular lipid droplets form a core region that is surrounded

by a cap of smooth muscle cells and a collagen rich matrix (69). The plaque can progress into an even more complex lesion in which the lipid core is surrounded by a fibrous cap. T-cells and macrophages infiltrate throughout the lesion and are particularly abundant in the shoulder region of the plaque and at the interface between the cap and the core. The inflammatory cells produce proteases, pro-inflammatory cytokines and protrombotic factors which destruct collagen and inhibit smooth muscle growth (70). The destabilized fibrous cap might fissure, usually at the shoulder region, and reveal thrombogenic plaque material. The exposed plaque material induces formation of a thrombus and consequently a clinical cardiovascular event.

1.4.3 TNF- α

TNF- α (also called TNF) is a pro-inflammatory cytokine which is produced predominantly by activated macrophages and Th1 cells (71). TNF- α is an early and important trigger for downstream mechanisms and acts within a complex network of cells and mediators of inflammation. TNF- α is initially expressed in the plasma membrane (transmembrane TNF- α) but can be released in a soluble form (soluble TNF- α) after enzymatic cleavage. Soluble TNF- α and transmembrane TNF- α are both biologically active. TNF- α binds to TNF- α receptor-1 and -2 (TNFR1 and TNFR2) (72). TNFR1 is constitutively expressed on virtually all nucleated cells, whereas TNFR2 is generally inducible and is preferably expressed on endothelial and hematopoietic cells (71). The effects of soluble TNF- α and transmembrane TNF- α can be pro-inflammatory through activation of NF- κ B, which leads to enhanced expression of pro-inflammatory cytokines, or lead to apoptosis, depending on the metabolic state of the cell (73). These pro-inflammatory and apoptotic pathways are mainly mediated through TNFR1. The consequences of TNFR2 signaling have been less well described but seem to promote cell proliferation, angiogenesis and NF- κ B activation (72).

TNF- α is usually not detectable in healthy individuals, but elevated serum and tissue levels are found in several inflammatory and infectious conditions (74;75). At low concentrations in tissues, TNF- α is thought to have beneficial effects, such as augmentation of host defense mechanisms against infections. At high concentrations, TNF- α can lead to a too excessive inflammatory response and consequently organ damage.

TNF- α has during the last decades been demonstrated to have a pivotal role in RA pathology (76). Activated macrophages are an important source of TNF- α in inflamed synovial tissue, and both the number of macrophages and the degree of TNF- α expression have been shown to

correlate with clinical measures of RA activity (77). TNF- α produced by the cells in the inflamed tissues also have been demonstrated to influence osteoclast differentiation and may thus promote bone destruction (10). Furthermore, transgenic mice expressing high levels of human TNF- α have been demonstrated to develop an inflammatory arthritis that resembled RA and which could be prevented by administration of monoclonal antibodies to human TNF- α (78). Finally, treatment with antibodies against TNF- α has been shown to significantly improve disease activity in patients with RA (79). TNF- α is also demonstrated to have a central role in AS and PsA. The cytokine has been detected in sacroiliac joints in patients with AS (80) and in synovial fluid in patients with PsA (81), and plasma levels correlate with the disease activity in AS patients (82). In line with the results from studies in patients with RA, TNF- α inhibition has been demonstrated improve disease activity also in patients with AS and PsA (83;84).

Data also support a central role for TNF- α in atherosclerosis and heart failure. Increased circulating levels of the cytokine have been shown in patients with myocardial infarction and patients with heart failure and predict recurrent cardiovascular events (85;86). Inflammatory cytokines are produced in the failing heart and seem to contribute to cardiac dysfunction (87). In a study with apolipoprotein E deficient mice, which are prone to atherosclerosis, the mice also deficient in TNF- α developed significantly less atherosclerotic lesions than the single apolipoprotein E deficient mice (88). During development of vascular pathology and atherosclerosis (chapter 1.4), TNF- α promotes the endothelial cell activation (89) and augments the inflammatory process in the artery wall. Further effects of TNF- α on vascular pathology might include impairment of NO bioavailability with consequent promotion of endothelial vasomotoric dysfunction (90). TNF- α is also demonstrated to promote thrombosis by increased expression of pro-coagulant proteins such as tissue factor. Furthermore, TNF- α also has profound metabolic effects, including promotion of insulin resistance and dyslipidemia (34).

1.5 Anti-inflammatory pharmacotherapy and CVD

A natural consequence of the central role of inflammation in atherogenesis and vascular pathology would be a beneficial effect on CVD from anti-inflammatory treatment. Studies have demonstrated that statin (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor) treatment not only lowers lipid levels, but also CRP levels in both the general population and

in patients with RA (91;92). Additionally, lipid lowering treatment improved disease activity and arterial stiffness in patients with RA (91;93). However, specific anti-inflammatory treatment strategies have not yet been proven favorable with regard to cardiovascular outcome in the general population. Whether treatment that lowers disease activity in inflammatory arthropathies may influence CVD, has been debated. Although the anti-inflammatory effect might be beneficial, commonly used drugs have been reported to have pro-atherogenic side effects. Available data regarding the effect on CVD of anti-inflammatory therapy include studies in the general population and RA patients with glucocorticoids, methotrexate (MTX), non-steroidal anti-inflammatory drugs (NSAIDs), selective cyclooxygenase (COX)-2 inhibitors and TNF- α antagonists.

1.5.1 Glucocorticoids, MTX, NSAIDs, COX-2 inhibitors and CVD

Treatment with glucocorticoids, which is common in both RA, AS, PsA and several other non-rheumatic conditions, has been associated with increased risk of CVD, particularly heart failure, in two large studies in the general population (94;95). The cardiovascular risk was largest in the patient group taking the highest oral dose of glucocorticoids, whereas a topical, nasal and inhaled use did not seem to increase the risk. These results were also valid in sub analyses which included only patients with inflammatory arthropathies. Studies addressing the effect of glucocorticoids on arterial wall properties in patients with RA seem to support these findings. Kumeda et al and Park et al have both reported that low dose (≤ 10 mg prednisolone) oral glucocorticoid use was not associated with increased CIMT values (31;33). These findings were later repeated by Del Rincon et al (96). Furthermore, in del Rincon et al's cohort of RA patients, those in the highest tertile of lifetime glucocorticoid exposure (>16.24 mg prednisone daily) had an increased frequency of carotid plaques and lower-limb arterial incompressibility (96). In line with these results, a review of the safety data from randomized controlled trials (RCTs) of low dose glucocorticoid treatment (≤ 10 mg prednisolone equivalent daily) in RA concluded that such treatment did not increase the risk of cardiovascular events (97). Furthermore, using low dose glucocorticoids did not significantly affect blood pressures. Thus, the overall body of evidence indicates that moderate and high doses, but not low doses, of glucocorticoids are unfavorable with regard to CVD both in the general population and in RA patients.

MTX is an important drug in the treatment of RA, and is also used in PsA. Krause et al have demonstrated that patients who do not respond to MTX treatment have an increased all cause

mortality compared to both patients with response to the drug and the general population (98). In conflict with this result, Landewe et al have reported in a research letter the same year that RA patients with pre-existing cardiovascular disease who started MTX treatment had a higher risk of death during follow-up than other patients in their cohort (99). Treatment with MTX in patients without pre-existing CVD, however, was not associated with increased mortality. Later, Choi et al have shown that MTX use reduce all-cause mortality in 1240 patients with RA, and that the main reduction was because of a reduced cardiovascular mortality (100). This finding was later supported by another study, in which the risk of hospitalization for heart failure was reduced by treatment with MTX (101). With regard to the effect of MTX on vascular function and structure, previous results are limited. Two cross sectional studies have addressed this issue, and both have shown that current MTX use did not influence CIMT values (31;33). All together, existing results indicate a beneficial effect of MTX on CVD, although further studies are needed.

Conventional NSAIDs and selective COX-2 inhibitors are extensively used in inflammatory arthropathies, and are the cornerstone in the therapy of AS. Concerns about cardiovascular adverse events associated with use of these medications arose in the early 2000's. The VIGOR (Vioxx GI Outcomes Research) study, which compared upper gastrointestinal clinical events but also reported adverse cardiovascular events, of rofecoxib and naproxen in patients with RA, had indicated a significant 4-fold increased risk of acute myocardial infarction with rofecoxib compared to naproxen (102). An increased risk of acute myocardial infarction or thrombotic cardiovascular events with rofecoxib therapy was also noted in a 2004 meta-analysis and in a trial of rofecoxib versus placebo for prevention of adenomatous colonic polyps, the APPROVe (Adenomatous Polyp PREvention On Vioxx) study (103;104). Rofecoxib was withdrawn from the market in September 2004. The use of other non-naproxen NSAIDs and selective COX-2 inhibitors also seem to be associated with an increased risk of cardiovascular events (105;106). However, the absolute risk is low, particularly when low doses are used and in patients with low cardiovascular risk. Naproxen appears to be the safest with respect to such risk, also in patients with RA (107;108). Studies assessing the effect of NSAIDs and selective COX-2 inhibitors on vascular function are limited.

The majority of data on cardiovascular effects of anti-inflammatory treatment in patients with inflammatory arthropathies are from cohort and case-control studies. When interpreting these results, a general problem is that patients with higher disease activity, and probably larger

inflammatory burden, most likely would receive more active treatment. Thus, many of these studies are confounded by indication. Furthermore, patients often use combinations of different medications and some drugs only for short periods. It has also been speculated that some of the beneficial effects observed with TNF- α antagonist (chapter 1.5.2) and MTX might be a steroid saving effect. Thus, results from this kind of studies need to be adjusted for confounding factors and to be interpreted with caution.

1.5.2 Anti-TNF- α therapy and CVD

The TNF- α antagonists infliximab, etanercept and adalimumab, which were the TNF- α antagonists in regular use at the time of initiation of this thesis, are all effective therapeutic agents in the main inflammatory arthropathies, and have since their introduction dramatically improved the outcome in these diseases. Infliximab and adalimumab are monoclonal antibodies and etanercept is a TNF- α receptor Fc-fusion protein. Accordingly, the TNF- α antagonists have a somewhat different mode of action, but clinical studies indicate comparable efficacy of these drugs in RA (109). Although TNF- α antagonists first were introduced for the treatment of RA, their effectiveness with regard to health-related quality of life is at least as good in AS (110). Furthermore, anti-TNF- α therapy has been demonstrated to induce a rapid reduction in synovial inflammatory cells in both RA, AS and PsA (111-113).

Due to the demonstrated role of TNF- α in heart failure and the positive experiences from anti-TNF- α therapy trials in patients with RA (chapter 1.4.3), RCTs with etanercept and infliximab in patients with chronic heart failure were conducted in the beginning of the 2000's (114;115). Pilot studies had shown improvement in left ventricular ejection fraction after initiation of etanercept (116;117), and a small study had demonstrated that etanercept improved endothelial dysfunction in patients with advanced heart failure (118). However, the RCTs of etanercept in heart failure showed no benefit of this drug, and treatment with infliximab in the highest dose (10 mg/kg) significantly increased heart failure hospitalization and death from any cause (114;115). It has later been speculated that the doses of TNF- α antagonists, particularly the highest dose of infliximab, were too high and that the inclusion criteria were too broad with NYHA heart failure class varying from II-IV in the study population (119).

In patients with inflammatory arthropathies, no RCTs with TNF- α antagonists and cardiovascular endpoints have been conducted, and available results in this regard are from an observational study. Data from the Swedish biologics registry have indicated that anti-TNF- α

therapy reduces the incidence of first cardiovascular event in patients with RA (120). In line with this finding, Irace et al and Hürlimann et al have reported of improved endothelial function in patients with RA after initiation of anti-TNF- α therapy (27;121), and similar results have been reported in patients with systemic vasculitis (122). On the other hand, Van Doornum et al did not find any effect of TNF-antagonists on arterial stiffness assessed as the augmentation index (AIx, chapter 3.2.1) in their cohort of patients with RA (123). Knowledge about the duration of the possible favorable effect of TNF- α antagonists on vascular function in inflammatory arthropathies is limited. Although the endothelial function improved already the day after drug infusion in the study by Irace et al, the improvement was temporary, and all vascular parameters returned to baseline levels before the next infusion at weeks 2 and 6 (27). In accordance with this finding, Gonzales-Juanatey et al have shown a similar response in endothelial function after infliximab infusions in patients with RA who had been treated with infliximab for a minimum of a year (124). Thus, available data indicate a beneficial effect of anti-TNF- α therapy on endothelial function, but the long-term outcome and data on possible effects on arterial stiffness and CIMT are missing.

1.6 *Calprotectin*

Calprotectin is a heterodimer compounded by the S100A8 and S100A9 proteins, also referred to as Myeloid Related Proteins (MRP) 8 and 14 or calgranulin a and b (125). Calprotectin is mainly expressed in cells of myeloid origin, particularly in neutrophils, monocytes and macrophages in early differentiation states (126), and appears to have anti-microbial properties and a regulatory function in infections. Intracellular calprotectin plays an important role in myeloid cell maturation, cell trafficking and arachidonic acid metabolism (127). Activated phagocytes secrete the heterodimer (128), which induces chemotaxis and amplifies the pro-inflammatory cascade through interaction with surface receptors on target cells. Possible receptors for calprotectin include the receptor for advanced glycation end products (RAGE), heparin sulfate proteoglycans and carboxylated glycans (127). The intracellular signaling pathways activated by ligation of these surface proteins by calprotectin are not yet fully elucidated, but with respect to RAGE, the main pathway includes activation of NF- κ B, which leads to enhanced expression of pro-inflammatory cytokines (127).

Several inflammatory conditions are associated with high calprotectin plasma levels (127). In inflammatory arthropathies, calprotectin is associated with the disease activity and synovial

inflammation (129-131), and anti-TNF- α therapy has been demonstrated to reduce both the number of infiltrating MRP-8 and MRP-14 expressing macrophages in synovial tissue and serum levels of calprotectin (131). Furthermore, data indicate a role for calprotectin also in vascular endothelial dysfunction and progression of atherosclerotic lesions. Viemann et al have demonstrated that calprotectin increased transcription of pro-inflammatory chemokines and adhesion molecules and induced loosening of endothelial cell junctions (132). McCormick et al have demonstrated high expression of the protein complex in human plaque, but not in normal artery intima, and also high expression in calcifying areas (133). Only one previous study has examined associations between circulating calprotectin levels, CIMT and carotid plaques in the general population (134). This study did find that carotid plaque occurrence, but not CIMT, was positively associated with plasma calprotectin levels. Possible associations between arterial stiffness and circulating calprotectin have not been previously reported.

1.7 ADMA

Asymmetric dimethylarginine (ADMA) is an endogenous NOS inhibitor which is derived during posttranslational modification of arginine residues on various proteins that are predominantly found in the cell nucleus (135). ADMA impairs NO synthesis by competing with L-arginine as a substrate for NOS (136), and the relationship between ADMA and L-arginine, usually expressed as the L-arginine/ADMA ratio, is suggested to be an important modulator of NOS activity (137;138). Intra-arterial infusions of ADMA or N-monomethylarginine (L-NMMA) have been demonstrated to reduce the endothelium dependent vasodilatation in the human forearm (139) and to increase aortic stiffness and AIX (140;141). L-NMMA is another endogenous NOS inhibitor which is produced and degraded by the same pathways as ADMA, and is known to affect endothelial function just as powerful as ADMA. However, since plasma ADMA levels are tenfold greater than plasma L-NMMA levels (136), the majority of studies considering cardiovascular risk have focused on ADMA and the L-arginine/ADMA ratio (138). Plasma and serum ADMA levels have been demonstrated to be associated with cardiovascular risk factors such as hypertension (142), hypercholesterolemia (137), diabetes mellitus (143) and renal failure (144). ADMA was first demonstrated in patients with chronic renal failure by Vallance and coworkers (136), and have since then been shown to be an independent predictor of overall mortality and cardiovascular outcome in this population (145). Later reports have indicated that circulatory

ADMA levels also predict myocardial infarctions and cardiovascular events in patients with coronary artery disease (146;147) and recurrent cardiovascular events after percutaneous coronary intervention in patients who had presented with an acute coronary syndrome (148). The main catabolic pathways for ADMA are renal excretion and metabolism to L-citrulline by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). Reduced renal excretion of ADMA in renal failure patients might explain some of the observed the increased cardiovascular risk in these patients. A previous in vitro study has shown that TNF- α increases ADMA concentration in endothelial cells by reduction of the activity of DDAH (149). The activity of DDAH strongly affects plasma ADMA levels (150). Circulating ADMA levels in patients with inflammatory arthropathies were scarcely examined at the time our project was designed. However, reduced endothelial function assessed as flow mediated dilatation (FMD) has been reported in these patients, and FMD has been demonstrated to improve during anti-TNF- α therapy (chapter 1.2, 1.3 and 1.5.2). Thus, increased ADMA levels induced by TNF- α 's effect on DDAH might contribute to the vascular pathology seen in inflammatory arthropathies, and anti-TNF- α therapy might be beneficial in this aspect by antagonizing this effect.

2 Aims

2.1 *General aim*

The general aim of this thesis was to examine the vascular effects of anti-TNF- α therapy in patients with inflammatory arthropathies, and to explore possible associations between changes in vascular function and structure and inflammatory markers.

2.2 *Specific research aims*

1. To explore the acute effects of infliximab infusions on arterial stiffness in patients with inflammatory arthropathies who are on long-term anti-TNF- α therapy (paper 1).
2. To assess the short- and long-term effects of initiation of anti-TNF- α therapy on arterial stiffness in patients with inflammatory arthropathies (paper 2 and paper 3).
3. To examine the effect of initiation of anti-TNF- α therapy on CIMT in patients with inflammatory arthropathies (paper 3).
4. To assess the associations between circulatory inflammatory markers (CRP, erythrocyte sedimentation rate [ESR] and calprotectin), arterial stiffness (paper 1, paper 2 and paper 3) and CIMT (paper 3).
5. To explore possible associations between measures of disease activity and arterial stiffness (paper 1, paper 2 and paper 3) and CIMT (paper 3).
6. To assess the effect of anti-TNF- α therapy on circulating ADMA, L-arginine and the L-arginine/ADMA ratio, and whether possible changes in ADMA or L-arginine/ADMA are associated with arterial stiffness or CIMT (paper 4).

3 Patients, design and methods

3.1 *Patients and design*

3.1.1 Paper 1

Seventeen patients with RA, AS or PsA and age > 18 years were recruited between February 2006 and May 2006 from Diakonhjemmet Hospital (Oslo). All patients had been treated with infliximab for at least 12 months before inclusion. No control patients were recruited. The blood pressure of patients with arterial hypertension, defined as systolic pressure ≥ 140 mmHg, diastolic pressure ≥ 90 mmHg or current use of antihypertensive medication, had to be well controlled ($< 140/90$ mmHg) for at least 6 months before the study start. Patients on lipid lowering treatment could only be included if they had been on a stable dose at least 6 months prior to inclusion. Treatment with antihypertensive or lipid lowering medication had to remain unchanged during the study period. Patients who initiated treatment with other biological disease-modifying anti-rheumatic therapy such as treatment targeting B-cells (rituximab), T-cells (abatacept), interleukin-1 (anakinra) or interleukin-6 (tocilizumab) would be excluded. Further exclusion criteria were permanent cardiac arrhythmia or previous carotid surgery.

The patients underwent arterial stiffness measurements, blood sampling and assessment of disease activity immediately before an infliximab infusion, and thereafter every 10th day until their next infusion. Infusion intervals of infliximab were determined by the patients' clinical response to the treatment and varied from every 4th week to every 8th week.

3.1.2 Paper 2, 3 and 4

Patients with RA, AS or PsA and age > 18 years who were scheduled to initiate anti-TNF- α therapy due to the activity of their rheumatic disease were eligible for inclusion in the studies reported in paper 2, 3 and 4. Exclusion criteria were as for the patients in paper 1 (chapter 3.1.1). Additionally, in paper 4 also patients with reduced kidney function or diabetes mellitus would be excluded. Sixty patients were recruited between June 2006 and January 2008 from Diakonhjemmet Hospital (Oslo) and Martina Hansens Hospital (Akershus). Fifty-eight of the 60 patients included in the studies were TNF- α antagonist drug naïve; two patients had previously (> 18 months before inclusion) used a TNF- α antagonist, but had ended treatment

due to failure or allergic reaction. The patients were asked about a possible study participation after their clinical indication for therapy had been established, but prior to the pre-treatment examinations (e.g. Mantoux' test, screening for heart failure and hepatitis). We expected that some of the patients would have to postpone therapy initiation due to findings in their pre-treatment examinations that required further evaluation, their working situation or planned operations. We had pre-specified that these patients would serve as controls for the total follow-up period or until they started with a TNF- α antagonist. Follow-up was one year after initiating anti-TNF- α therapy, or one year from the baseline examination for the control patients who did not initiate this therapy. The patients in the control group were not scheduled to receive specified alternative treatment, and each patient's treating rheumatologist was free to adjust medication in accordance with the disease activity.

In paper 2, we reported the short-term (3 months) effects of anti-TNF- α therapy on arterial stiffness. Because we in this thesis intended to explore both the short-term and the long-term (one year) effects of anti-TNF- α therapy on arterial stiffness, and since we expected that several of the patients in the control group would start therapy after a couple of months, we had pre-specified an evaluation of changes in arterial stiffness after 3 months. At this time point, none of the patients had dropped out, and the 35 patients who had started with anti-TNF- α therapy were compared to a non-treatment group of 25 patients. Both the anti-TNF- α group and the control group underwent assessments of arterial stiffness and disease activity and blood sampling at baseline and after 3 months.

Five patients terminated the study before a follow-up after one year. Three patients were excluded because they initiated treatment with rituximab or anakinra. One patient ended anti-TNF- α therapy because of suspected cancer, and another because of an allergic reaction. Thirty-six patients continued anti-TNF- α treatment for one year, and 19 patients remained without anti-TNF- α treatment during a total follow-up of one year. All these patients underwent measurements of arterial stiffness, disease activity and blood sampling at baseline and every third month until one year. Additionally, CIMT was assessed at baseline, 6 months and one year. In paper 3 we reported the effects of one-year anti-TNF- α therapy on arterial stiffness, CIMT and circulating calprotectin. In paper 4 we reported the effects of anti-TNF- α therapy on ADMA, L-arginine and L-arginine/ADMA, which were analyzed from plasma samples taken at the baseline, 3 and 12 months visits.

3.2 *Methods*

3.2.1 *Arterial stiffness*

Arterial stiffness can be assessed as local, regional or systemic stiffness with a large selection of devices (20). We measured the carotid-femoral pulse wave velocity and the AIx with the Sphygmocor device version 7.1 (AtCor Medical, Sydney, Australia) and a validated tonometer (SPC-301; Millar instruments, Houston, USA). Arterial stiffness examinations were conducted in concordance with the standardizations recommended in the expert consensus document on arterial stiffness (20).

Carotid-femoral pulse wave velocity is recognized as a measure of aortic pulse wave velocity (aPWV), and is currently accepted as the gold standard measurement of arterial stiffness (20). aPWV is the speed at which the pulse pressure wave travels down the aorta, and is thus an assessment of regional aortic stiffness. For the evaluation of aPWV, carotid and femoral artery waveforms were sequentially recorded with tonometry and the transit time was calculated by the integrated software using simultaneously recorded ECG as reference (151). The wave travel distance was obtained by subtracting the distance from the carotid location to the sternal notch from the distance between the sternal notch and the femoral site of recording (20). aPWV has been demonstrated to be a predictor of CVD in several patient groups with different cardiovascular risk factors such as hypertension, diabetes and hypercholesterolemia and in the general population (20). Increased stiffness of the aorta and carotid arteries have previously been demonstrated in patients with RA (23;24). Furthermore, aPWV has been demonstrated to change both during anti-inflammatory treatment in patients with vasculitis (122) and as a response to a mild systemic inflammation induced by vaccination in a healthy group of subject (38). The change in aPWV in the latter study was measurable already eight hours after the vaccination, thus demonstrating that aPWV may change rapidly in relation to an inflammatory stimulus (38).

AIx is a measure of pulse wave reflection (20). The arterial pressure waveform is considered to be a composite of the forward pressure wave generated by the left ventricular contraction and the waves reflected from the arterial periphery. AIx refers to the difference between the second and the first systolic peaks of the waveform, expressed as a percentage of the pulse pressure (20). AIx is dependent on the magnitude and site of pulse wave reflection and the speed of the reflected wave. Thus, AIx is influenced by arterial stiffness, but also peripheral

vascular resistance (152). To obtain the AIx, peripheral pressure waveforms were recorded from the radial artery at the wrist with the tonometer. Corresponding central aortic waveforms were generated by integrated software from which central hemodynamics and aortic AIx were calculated (153). AIx has been demonstrated to be a predictor of all-cause mortality in patients with kidney failure and of cardiovascular events in patients with hypertension, stable angina or acute coronary syndromes (20). AIx has been shown to be increased in patients with RA compared to healthy controls (22), and to be a more sensitive measure of vascular dysfunction than brachial FMD in this patient population (25).

3.2.2 Carotid intima media thickness

CIMT is a measure of the combined thickness of the intima and media layers, the two layers closest to the vessel lumen, in the carotid artery. We measured CIMT in the common carotid artery on both sides of the neck. The common carotid artery was visualized in a longitudinal view and scanned for plaques before CIMT was assessed. CIMT measurements were performed in the far wall of approximately 2 cm long segments free of plaques in the distal common carotid artery close to the carotid bulb (154). During examination, the patient's neck was slightly hyperextended and the head rotated in the opposite direction of the probe. To standardize the position of measurements at the consecutive visits a protractor frame was used. CIMT was assessed with the multiarray echotracking system Art.Lab (Esaote, Maastricht, the Netherlands) equipped with a 5-10 MHz linear array transducer (155). The system utilizes rough radio frequency data to automatically register intima media thickness of the common carotid artery for each subsequent cardiac cycle over a period of 6 seconds (156). The rough radio frequency data were analyzed both online and from the 6-second stored cine-loops.

CIMT has been demonstrated in several studies to be a marker of the generalized atherosclerotic burden and a predictor of cardiovascular disease (154;157). Furthermore, CIMT is a validated surrogate endpoint for cardiovascular disease in intervention trials, particularly in studies with statins or anti-hypertensive medication (64;158;159). CIMT has been demonstrated to be associated with markers of systemic inflammation both in the general population and in patients with RA (32;40;160), but results regarding an effect of anti-inflammatory treatment on CIMT progression have been missing. On average, the follow-up time before an observable change in CIMT after initiation of medical treatment seems to be one to two years (64). However, a small study exploring the effect of statin treatment on

carotid and femoral artery intima media thickness has demonstrated significant changes already after 8 weeks of treatment (161). The authors of this study attributed the rapid improvement in carotid and femoral intima media thickness to the anti-inflammatory effect of statins.

3.2.3 Disease activity

The clinical status and disease activity were evaluated according to the American College of Rheumatology's preliminary core set of disease activity measures for RA clinical trials (162). Our assessments included counts of tender and swollen joints, measurement of acute phase reactants (ESR and CRP), physician's evaluation of global disease activity on a 100 mm visual analog scale (VAS), and the patients' assessment of pain and global disease activity on 100 mm VASs and evaluation of physical function with the Health Assessment Questionnaire (HAQ). We also obtained the patients' assessment of fatigue on a 100 mm VAS. The disease activity score based on 28 joints (DAS 28) and ESR ($\text{DAS28} = 0.56 \sqrt{[\text{number of tender joints}] + 0.28 \sqrt{[\text{number of swollen joints}] + 0.70 \ln [\text{ESR}] + 0.014 [\text{patient's evaluation of global disease activity on a VAS}]}$) (163) was computed in the patients with RA.

3.2.4 Laboratory Measurements

Fasting blood samples were collected at all visits. ESR was analyzed by the Westergren method. Hemoglobin (Hb), total cholesterol, LDL, high density lipoprotein cholesterol, triglycerides, CRP and creatinine were determined by standard methodology at the time of examinations (Sysmex Corporation, Kobe, Japan and Roche Diagnostics GmbH, Mannheim, Germany). For analyses of calprotectin, ADMA and L-arginine, EDTA plasma samples were stored at -80°C and thawed at room temperature before the analyses. Calprotectin was determined with ELISA kits from Calpro AS, Lysaker, Norway. L-arginine and ADMA were determined by high performance liquid chromatography and precolumn derivatization with o-phthalaldehyde (Sigma Chemicals Co, St.Louis, MO).

3.2.5 Statistics

Continuous data are presented as mean with standard deviation (SD), median with quartile cut-points (25th and 75th percentiles) or geometric means with 95% confidence intervals as appropriate. Between group differences were compared using Student's t-test for independent

samples or with Mann-Whitney U test when data were skewed. Within group changes were assessed by paired Student's t-test or Wilcoxon signed-rank test. Categorical variables are expressed as numbers and were compared using Pearson's χ^2 test. Bivariate relations were analyzed using Pearson's, or in the case of skewed variables, Spearman's correlation coefficient.

Analyses of covariance (ANCOVA) were used to in paper 2 to explore associations between the baseline value and change in aPWV with CRP and change in CRP as both a continuous variable and categorized into quartiles. In paper 4, baseline associations with aPWV were determined with multivariable linear regression analyses.

For determinants of change in aPWV in paper 2, a robust multivariable regression analysis was performed, because of non-normality of the residuals.

Multivariable mixed model repeated measure linear regression analyses were used to examine associations between aPWV, AIx, CIMT, ADMA, L-arginine/ADMA, markers of inflammation and disease activity in paper 1, 3 and 4. The effects of anti-TNF- α therapy in paper 3 and 4 were examined as the interaction between the variables anti-TNF- α therapy and time. Mixed model repeated measures analysis is a longitudinal linear regression analysis that controls for multiple testing of the same patient by modeling the covariance between the repeated measurements of each individual as a clustered random effect. An unstructured covariance matrix was used in our analyses assuming that the correlation for each level of within-subject factor was different. The mixed model procedure deals with missing values by assuming them to be missing at random without removing the individual from the dataset.

Bivariate analyses of clinically important covariates were performed, and covariates with P-value < 0.25 , or covariates known to influence the respective dependent variables, were entered into the multivariable models. Variables were then removed in a step-down manner according to levels of significance as described by Hosmer and Lemeshow (164). The models were examined for relevant interactions and confounding in a standard manner.

Due to the non-randomized setting of our studies, the multivariable analyses in paper 2 and 3 were repeated after a one to one matching of the patients in the control group with a similar numbers of patients in the treatment group by age, sex and mean arterial pressure (MAP). Age, sex and blood pressure are some of the most important predictors of aPWV, AIx and CIMT (20;154). In the analysis of the matched pairs in paper 2, a two-way analysis of

variance (ANOVA) with randomized blocks was applied. In paper 3, the mixed models analyses were repeated after matching with the matched pairs included as a random factor.

Standardised response mean values (mean change from baseline / SD of the change) were used to illustrate the magnitude of the response in key variables after three 3 months in paper 2. Standardised response mean values were interpreted as effect sizes according to Cohen (165); standardised response means ≥ 0.2 and < 0.5 , ≥ 0.5 and < 0.8 , and ≥ 0.8 indicate small, moderate and large magnitudes of change, respectively.

All statistic tests were two-sided and P-values ≤ 0.05 were considered significant. Statistical analyses were performed with Statistical Package for the Social Sciences (SPSS), version 16.0-18.0 (SPSS Inc., Chicago, USA) and R (R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). A statistician was consulted regarding the statistical analyses in this thesis.

3.3 *Ethical aspects*

Approval was obtained from the regional research ethics committee, and written informed consent was obtained from each participant. The Norwegian social science data services gave permission to the storage of research data, and authorization to store biological material was granted by the Directorate of Health. All studies were performed according to ethical principles of the declaration of Helsinki.

4 Summaries of results

4.1 *Paper 1*

Changes in arterial stiffness during continued infliximab treatment in patients with inflammatory arthropathies.

The aim of this study was to evaluate arterial stiffness and disease activity between two infusions of infliximab in patients with inflammatory arthropathies who had been treated with this drug for at least 12 months prior to inclusion in the study. AIx, aPWV and disease activity were measured in 17 patients immediately before an infliximab infusion and thereafter every 10th day until their next infusion scheduled at week 4-8. AIx and aPWV did not change during the period between the infliximab infusions. The patients had a temporary improvement in the global disease activity assessed on VAS by the patients ($P=0.04$) and the investigator ($P=0.02$) after the infusion. In the group of patients with RA, the DAS28 changed in a similar manner ($P=0.003$). CRP and ESR remained unchanged. Multivariable repeated mixed model analyses showed no longitudinal associations between AIx, aPWV, measures of disease activity, CRP or ESR. These findings were confirmed with analyses on the individual level with line charts.

4.2 *Paper 2*

Tumor Necrosis Factor- α antagonists improve aortic stiffness in patients with inflammatory arthropathies: A controlled study.

The purpose of this study was to evaluate the effect of treatment with TNF- α antagonists on arterial stiffness after three months in patients with inflammatory arthropathies. Changes in aPWV and AIx were examined in 35 patients who had started with anti-TNF- α therapy and in 25 non-treated patients. aPWV (mean [SD]) was reduced in the treatment group, but not in the control group (-0.50 [0.78] m/s versus 0.05 [0.54] m/s, respectively; $P=0.002$). Reductions in aPWV were seen in both the patients with RA, AS and PsA and in subgroups according to use of various TNF- α antagonists. Central pressures and AIx remained unchanged in both the treatment and the control group. CRP and DAS28 were significantly reduced in the treatment group (-9.3 [20.2] mg/L, $P<0.001$ and -0.74 [0.91], $P=0.004$). In an ANCOVA analysis with change in CRP categorized into quartiles, patients with the largest reduction in CRP had a

significant greater reduction in aPWV after 3 months than the patients in the lowest quartile (-0.63 [0.75] m/s versus 0.06 [0.60] m/s, respectively; $P=0.01$), but the overall model was not significant ($P=0.08$). The change in aPWV and the inflammatory markers in the two patient groups were further compared with standardised response means, which demonstrated a moderate-to-high responsiveness (standardised response mean >0.50) in aPWV, CRP, ESR, and DAS28 to anti-TNF- α therapy. In a multiple linear regression model only treatment with TNF- α antagonist ($\beta=-0.485$, $P=0.003$) and change in MAP ($\beta=0.029$, $P=0.008$) were associated with alterations in aPWV. In a repeated analysis after matching patients in the two groups by sex, age and MAP, anti-TNF- α therapy remained significantly associated with change in aPWV ($\beta=-0.675$, $P=0.0045$).

4.3 *Paper 3*

Effect of 1-year anti-TNF- α therapy on aortic stiffness, carotid atherosclerosis and calprotectin in inflammatory arthropathies: A controlled study.

The objective of this study was to examine the effect of one year of treatment with TNF- α antagonists on arterial stiffness and CIMT progression in patients with inflammatory arthropathies, and possible associations to calprotectin. Fifty-five patients were followed with regular examinations for one year. Thirty-six patients starting with anti-TNF- α therapy were compared with a non-treatment group of 19 patients. After one year, aPWV (mean [SD]) was improved in the treatment group, but not in the control group (-0.54 [0.79] m/s vs. 0.06 [0.61] m/s, $P=0.004$), and CIMT progression (median [quartile cut-points, 25th and 75th percentiles]) was reduced in the treatment group compared to control group (-0.002 [-0.038 , 0.030] mm vs. 0.030 [0.011 , 0.043] mm, $P=0.01$). The main change in aPWV occurred the three first months after initiation of anti-TNF- α therapy, but aPWV remained at a stable lower level in the treatment group compared to the control group during the entire follow-up period. Measures of inflammatory activity including calprotectin improved significantly in the treatment group. In multivariable repeated mixed model analyses, anti-TNF- α therapy over time was associated with improved aPWV ($P=0.02$) and reduced CIMT progression ($P=0.04$), and calprotectin was longitudinally associated with aPWV ($P=0.02$). The results from the multivariable analyses did not change after matching the patients for age, sex and MAP.

4.4 Paper 4

The L-arginine/asymmetric dimethylarginine ratio is improved by anti-Tumor Necrosis Factor- α therapy in inflammatory arthropathies. Associations with aortic stiffness.

The purpose of this study was to examine the effect of treatment with TNF- α antagonists on ADMA and L-arginine/ADMA in patients with inflammatory arthropathies. Furthermore, we sought to explore possible associations between ADMA, L-arginine/ADMA, arterial stiffness and CIMT. Thirty-six patients who started with anti-TNF- α therapy and continued this treatment for one year were compared with a non-treated group of 19 patients. Plasma ADMA and L-arginine, aPWV and AIx were assessed at baseline, 3 and 12 months. CIMT was measured at baseline and 12 months. Anti-TNF- α therapy for 12 months increased the L-arginine/ADMA ratio (mean [SD]) in the anti-TNF- α group compared to the control group (9 [30] vs. -7 [22], $P=0.03$), but did not change ADMA (0.01 [0.09] $\mu\text{mol/L}$ vs. 0.00 [0.09] $\mu\text{mol/L}$, $P=0.88$). Baseline aPWV was associated with ADMA ($P=0.02$) and L-arginine/ADMA ($P=0.02$) in multiple linear regression analyses, and the L-arginine/ADMA ratio was continuously associated with aPWV after initiation of anti-TNF- α therapy in a multivariable mixed analysis ($P=0.03$). L-arginine, ADMA and L-arginine/ADMA were not associated with any of AIx, central blood pressures or CIMT.

5 General discussion

5.1 *Methodological considerations*

5.1.1 Study design and patients

The knowledge about the natural development in measures of vascular function and structure in patients with inflammatory diseases over time, and also how individual fluctuation in disease activity would affect these measures, were scarcely examined at the time we conducted our studies. We therefore included a control group of patients who did not receive anti-TNF- α therapy but who had similar inflammatory activity and indication for this therapy as those in the treatment group for the studies reported in paper 2, 3 and 4. Obviously, the optimal design would have been to randomize to anti-TNF- α therapy or placebo. However, we did not find this design ethically acceptable as all patients had a clinical indication for treatment with TNF- α antagonists according to accepted recommendations for such treatment (166). Only one previous study exploring vascular effects of anti-TNF- α therapy in patients with inflammatory arthropathies has applied a randomized design (167). In that study, all patients in the non-TNF- α antagonist group had switched to the treatment group before the vascular evaluation was performed. This observation illustrates the difficulties of conducting a randomized study with anti-inflammatory treatment assessing cardiovascular endpoints in patients with inflammatory arthropathies. Our choice of control group could have introduced potential bias, i.e. such as a tendency to more active disease in the treatment group or a higher rate of co-morbidities in the control group. However, we could not detect that any of these factors influenced our results. The treatment group and the control group were recruited from the same clinical setting and the two patients groups did not differ with regard to demographic characteristics, baseline disease activity or co-morbidities (paper 2, 3 and 4). Nevertheless, we chose to repeat the statistical multivariable analyses after matching patients in the two treatment groups by sex, age and MAP. New analyses after matching for these parameters did not meaningfully change the results (paper 2 and 3).

We included three types of inflammatory arthropathies in our studies. RA, AS and PsA are all associated with an increased cardiovascular risk, and good efficacy of anti-TNF- α is demonstrated in all diseases. Whereas the CVD risk in RA has been known for some years (chapter 1.2), the cardiovascular co-morbidity in AS and PsA has gained increasing attention the recent years. Excess risk of myocardial infarction has been demonstrated in both

conditions (168-170), and Hollan et al have reported that a diagnosis of AS or PsA was a stronger predictor for early coronary artery bypass grafting than most traditional cardiovascular risk factors (171). Accordingly, several studies have shown increased levels of vascular dysfunction in AS and PsA (48;172-177), whereas results regarding CIMT have been more inconsistent (48;173;176;178-185). The prevalence of CVD in AS and PsA has in one study been shown to resemble that of RA (186), whereas others report of a somewhat lower risk in AS and PsA compared to RA (170). These results indicate that an increased cardiovascular risk and premature vascular pathology not are restricted to RA, but also applies to AS and PsA. Recent studies have confirmed an equally good efficacy of anti-TNF- α therapy with regard to health related quality of life and drug retention rates in AS and PsA compared to RA (187-189). Anti-TNF- α therapy does not seem to have the beneficial effect on new bone formation in AS which has been demonstrated for the erosive bone destruction in RA and PsA (190). However, a rapid effect on joint inflammation and cellularity (chapter 1.5.2) and circulatory inflammation, which probably are more important with regard to vascular pathology, is demonstrated in both RA, AS and PsA (191). Nevertheless, including RA, AS and PsA made the total population more heterogeneous, and the diseases have differences in both treatment options (chapter 1.5) and disease pathology (chapter 1.1), which could have influenced our results. The effects of anti-TNF- α therapy on the vascular endpoints in our studies were comparable and the reductions in aPWV were significant within the different diagnose groups (paper 2 and 3). Furthermore, the multivariable statistical analyses did not reveal that type of rheumatic diagnosis or TNF- α antagonists influenced our results (paper 1, 2 and 3). Nonetheless, this project was not powered or designed to examine differences between the RA, AS and PsA subgroups and significant differences between these groups cannot be fully excluded. As illustrated in the online supplementary information file 3 to paper 3, AS patients seemed to have a smaller reduction in aPWV than patients with RA and PsA, although the changes in the three groups were not significantly different. However, the AS patients in the treatment group in paper 3 were younger and had lower blood pressure than the RA and PsA patients (unpublished data). As recently reported by *the reference values for arterial stiffness collaboration*, age and blood pressure are the main determinants for aPWV (192). Age and blood pressure were significantly associated with aPWV also in our population (paper 3). Therefore, differences in age, baseline blood pressure and aPWV may explain a possible unlike effect of anti-TNF- α therapy on aPWV in AS patients compared to RA and PsA patients. The possible difference in the change in aPWV could also be explained by a lower baseline inflammatory activity in the AS patients compared to the RA and PsA

patients. The patients with AS could have initiated anti-TNF- α therapy at lower disease inflammatory activity than patients with RA or PsA due to lack of other therapeutic options (chapter 1.5). In the treatment group, patients with AS had lower calprotectin levels than both RA and PsA patients, similar ESR as PsA patients but lower than RA patients, and similar CRP values as both RA and PsA patients (unpublished data). Consequently, including patients with AS might have reduced the beneficial effect on vascular parameters, but also these patients had a significant reduction in aPWV and a similar timing of the change as the RA and PsA patients (paper 2 and 3).

We also included three different types of anti-TNF- α treatment in the studies reported in papers 2, 3 and 4 (chapter 3.1.2), and similar objections may be raised against this choice as to the inclusion of different types of inflammatory arthropathies. The numerical effect of the different TNF- α were similar, and multivariable statistical analyses did not reveal that type of TNF- α antagonists influenced our results (paper 2 and 3).

5.1.2 Methods

Different techniques and devices can be used both for arterial stiffness and CINT measurements, and methodological choices clearly influence the obtained values (154;192). We measured aPWV using the pulse wave travel distance obtained by the subtraction method, which give results that differ from results obtained with other ways of assessing the pulse wave travel distance. Furthermore, our CINT values were calculated automatically and given as the average CINT during the whole cardiac cycle, and may thus differ from values obtained with manual or semi-automatic reading or in end diastole (193). These aspects are of special importance when aPWV and CINT values are compared cross-sectionally with previous obtained values. Our primary endpoints were, however, the changes in the vascular parameters. Since these methodological differences are constant between each visit, they are of less importance in the interpretation of longitudinal results.

All vascular measurements reported in this thesis were done by the same examiner and with identical devices at every visit. Our repeatability results demonstrated a high intra-reader correlation coefficient, acceptable limits of agreement and consistent Bland Altman-plots for repeated measures of AIx, aPWV and CINT (paper 3).

Infliximab, etanercept and adalimumab are administered with different time intervals.

Infliximab is after the two first months usually given every 8th week, etanercept once or twice

weekly and adalimumab every other week. Therefore, the vascular examinations were conducted with different time relations to the last drug administration according to which drug the patients was using. In light of the previously demonstrated transient effect of infliximab on endothelial function (chapter 1.5.2) (27;124), this could have affected our results. We did, however, not demonstrate any effect of infliximab infusions on AIx or aPWV in our patients who were on long-term treatment and had stable low CRP and ESR levels (paper 1). Furthermore, any effects due to differences in timing between drug administrations and the vascular measurements would be by chance and would not systematically affect the results.

DAS28, VASs and HAQ are all important outcome measures in clinical trials in patients with inflammatory arthropathies. However, these measures may be influenced by co-morbidities, personal factors, pain and chronic joint damage, and thus not only reflect the present inflammatory activity of the rheumatic disease (194). Furthermore, particularly the patient reported outcomes may have been influenced by the open label design of our studies.

Disease activity scores specific for AS and PsA equivalent to the DAS28 in RA were not reported in this thesis. The motivation for this choice was an intention to focus on disease activity measures that were assessable in all three groups of patients due to the small patient populations. Nevertheless, we reported the DAS28 in patients with RA since this was the largest group of patients and because this measure had been reported in the previous studies examining responses in vascular function to anti-TNF- α therapy (27;121). Importantly, the latest composite scores for assessment of AS and PsA were not available when this research work was started (195-197).

5.2 Discussion of main findings

5.2.1 The effect of TNF- α antagonists on vascular function and structure

We showed in our studies that TNF- α antagonists have a beneficial effect on both vascular function (aPWV, the L-arginine/ADMA ratio) and vascular structure (CIMT) (paper 2, 3 and 4) in patients with inflammatory arthropathies.

With regard to effects of anti-TNF- α therapy on vascular function, other groups have reported findings in line with ours during the recent years (167;177;198-201). The majority of these studies have assessed the effect on FMD, which is a measure of endothelial function, whereas only two studies besides ours have explored changes of anti-TNF- α therapy on aPWV

(167;200). Maki-Petaja et al demonstrated a reduction in aPWV after twelve weeks of therapy in a group of nine patients with RA (200) and Wong et al showed an improvement in aPWV in sixteen RA patients after 56 weeks of anti-TNF- α therapy (167). To our knowledge, data on anti-TNF- α therapy's effect on ADMA and L-arginine have been missing. We demonstrated that anti-TNF- α therapy increased plasma L-arginine and the L-arginine/ADMA ratio, but had no effect on ADMA (paper 4). These findings are in accordance with two recently published cross-sectional studies which have reported that current use of TNF- α antagonist did not influence plasma ADMA levels in patients with AS (202;203).

AIx, which is another measure of vascular function, did not change after initiation of anti-TNF- α therapy in our population (paper 2 and 3). Although Galarraga et al recently have reported improvement in AIx after two months of treatment with etanercept in patients with RA (204), our finding is in concordance with the majority of previous studies (123;167;184;200;205). One possible explanation to these observations is that the reduction in systemic inflammation induces peripheral vasoconstriction, or normalisation of the vasculature's tonus. Peripheral vasodilatation is a well-known feature of infectious and inflammatory diseases. The change in peripheral vascular tonus may result in pulse wave reflection points closer to the heart or change in the magnitude of wave reflection, and the consequent modifications in wave reflections would thus counteract the influence of the reduced aPWV on AIx. In line with this explanation, AIx has been demonstrated to decrease simultaneously with an increase in aPWV during a vaccination induced low-grade inflammation in a group of healthy subjects (38). Furthermore, infliximab infusions in patients with RA have been shown to reduce the brachial artery's diameter (27).

Current data on TNF- α antagonists' effect on vascular structure such as CIMT are more inconsistent than the results on vascular function. Wong et al, Sidiropoulos et al and Gonzalez-Juanatey et al did not find any effect of anti-TNF- α therapy on CIMT in patients with RA (167;201;206). In contrast, two larger studies, which included RA patients receiving standard therapy as controls, have shown a reduction in CIMT after such treatment (207;208). The majority of results from trials with statin and anti-hypertensive treatment and CIMT as endpoint indicate that the effect after medical intervention on CIMT most often is a reduced progression and not an actual reduction in the treatment group (158;159). These results emphasize the importance of a control group in studies evaluating CIMT, and might be a part of the explanation of the negative findings in the studies by Wong et al, Sidiropoulos et al and Gonzalez-Juanatey et al (167;201;206).

We demonstrated that aPWV was reduced in the treatment group at the first follow-up visit at 3 months (paper 2 and 3) and that this beneficial treatment effect was maintained throughout the one-year follow-up period (paper 3). *The reference values for arterial stiffness collaboration* has recently published pulse wave velocity normal and reference values according to age and blood pressure category (192). In our one-year follow-up study, the patients in the treatment group were on average 47.2 years old and their mean blood pressure was 131/79 mmHg (paper 3). According to the reference values the expected mean aPWV value in these patients would be 6.6-7.0 m/s. The mean aPWV in the treatment group after one year was 6.94 m/s. Thus, one possible interpretation of our results would be that anti-TNF- α therapy reduced aPWV to the expected level for a subject without systemic inflammation. Furthermore, this explanation is supported by our observation of the stable aPWV measurements in patients on long-term infliximab therapy (paper 1) and by Maki-Petaja et al's demonstration of a reduction in aPWV to the level of healthy controls after twelve weeks of anti-TNF- α therapy (200).

Structural alterations such as changes in CIMT during intervention trials are normally not detectable before one year of follow-up, and would be expected to follow a more regular pattern than changes in vascular function (64). The effect of treatment with a TNF- α antagonist on CIMT in our population was comparable with that reported in studies evaluating the efficacy of statin therapy on far wall CIMT (64). In a previous meta-analysis, statin therapy was associated with an average decrease in CIMT progression of 0.012 mm/year (158). CIMT increases with age, also in healthy subjects without risk factors for atherosclerosis (154), and a small progression in CIMT in the control group was expected. The observed increase in CIMT in the control group in our population was in the upper level of a normal increase after one year (64).

We showed in our population that baseline aPWV was associated with plasma ADMA and L-arginine/ADMA levels. Previous data on an association between the L-arginine/ADMA ratio and aPWV or AIx both in inflammatory arthropathies and other populations have been missing, whereas the existing results regarding relations between circulatory ADMA and aPWV or AIx have been inconsistent. AIx has been demonstrated to correlate with ADMA in patients referred to elective percutaneous coronary intervention and in the general population (209;210), whereas studies in hypertensive patients and another study in the general population demonstrated no relation (210-212). None of these studies have demonstrated a correlation between aPWV and ADMA. In patients with inflammatory arthropathies, one

study has reported of an inverse correlation between circulatory ADMA levels and coronary flow reserve (213), whereas another study has showed no association between brachial artery FMD and ADMA (202). However, the latter study included patients who were on treatment with a TNF- α antagonist. Anti-TNF- α therapy has been demonstrated to improve FMD (27;121;200), and inclusion of these patients may have reduced a possible correlation between FMD and ADMA. In our population, the greatest change in both aPWV and the L-arginine/ADMA ratio took place during the three first months after initiation of anti-TNF- α therapy (Paper 3 and 4), and the L-arginine/ADMA ratio was continuously associated with aPWV after initiation of TNF- α antagonists. Thus, improvement in NO availability subsequent to increase in the L-arginine/ADMA ratio may be involved in the favorable effect of anti-TNF- α therapy on aPWV in our population.

Previous studies indicate that circulating ADMA levels are associated with CIMT (211;214;215) and predict CIMT progression (214) in the general population, although a large cross sectional study within the Framingham heart study offspring cohort found significant associations only to internal carotid artery and carotid bulb intima media thickness and not to CIMT (216). In patients with inflammatory arthropathies, data regarding correlations between ADMA and CIMT are inconsistent (202;213;217). We did not observe any associations between CIMT and ADMA, L-arginine or the L-arginine/ADMA ratio. However, the patients in our study had CIMT values in the lower normal range. Therefore, correlations between CIMT and L-arginine/ADMA or ADMA may be easier to evaluate in patient groups with more pronounced atherosclerosis than in our cohort, and in larger patient groups.

Inflammation may promote vascular pathology and CVD through direct effects on the artery wall (chapter 1.4), but also by influence on traditional risk factors such as serum lipids, insulin resistance (37), or blood pressure (218). As discussed in chapter 1.2-1.3, inflammatory arthropathies are associated with increased levels of cardiovascular risk factors such as smoking, dyslipidemia, insulin resistance and, as recently reported, an increased prevalence of hypertension (219). Data regarding the effect of anti-TNF- α therapy on quantitative serum lipid levels have been inconsistent, particularly in long-term studies. It seems overall that this treatment improves the atherogenic index during the first months of therapy, but results obtained after longer treatment periods are divergent (220;221). Regarding the effect of anti-TNF- α therapy on blood pressure, Klarenbeek et al have recently published blood pressure data from the BeSTstudy in which RA patients were randomized to four different types of anti-rheumatic treatment strategies (222). One of these strategies included a TNF- α antagonist

(infliximab), and the results demonstrated that anti-TNF- α therapy had a favorable effect on blood pressures compared to the other anti-rheumatic therapy options. We did not find any significant changes in blood pressures or standard serum lipids in our studies. Thus, our findings might support previous results of a beneficial effect of anti-inflammatory treatment on vascular pathology beyond the influence on traditional cardiovascular risk factors in inflammatory arthropathies.

Altogether, available results indicate a beneficial effect of TNF- α antagonists on vascular function and structure in inflammatory arthropathies. This interpretation is in line with two recently published reviews and one American registry study of anti-TNF- α therapy's effect on CVD in patients with RA, which concluded that such therapy appears to have a favourable effect (223-225). However, most of the previous studies that have examined vascular effects of anti-TNF- α therapy have small patient numbers, which may raise the possibility of publication bias, and RCTs in this field are lacking (1.5.2).

5.2.2 Relations between vascular measurements and inflammatory markers

Vascular pathology has previously been demonstrated in cross sectional examinations to correlate with disease duration and/or markers of inflammation in patients with RA and in the general population (200;226;227). Our research group have recently demonstrated in another cohort of patients with RA that augmented CRP levels predicted increased aPWV and AIx (228), and that aPWV was lower in patients with low disease activity compared to those with active disease (229). As expected, the patients who started anti-TNF- α therapy in the present studies had a significant improvement in both biochemical inflammatory markers and clinical disease activity measures (paper 2 and 3). These findings are in accordance with previously published studies, which have demonstrated improvement in vascular function and structure concurrent with reduction in several inflammatory and disease activity markers such as CRP, ESR, DAS28 and patients' global assessment of disease activity on VAS (27;121;124;167;200;201;207;208;230).

We could not demonstrate any significant correlations between changes in aPWV or CIMT and CRP, ESR, DAS28, the VASs or HAQ (paper 1, 2 and 3). In concordance with our results, the majority of other studies also report of parallel reductions in measures of inflammatory activity and vascular pathology, but no correlations (27;121;124;167;200;201;208;230). The lack of associations in our studies may very well be

related to the size of the patient groups since changes in aPWV after three months of anti-TNF- α therapy showed a tendency to correlate with change in CRP during the same time frame (paper 2). In line with this interpretation, a recent review of articles addressing the association between CIMT and soluble markers of inflammation, endothelial damage and hemostasis concluded that of soluble biomarkers only CRP and fibrinogen seemed to be related to CIMT, but that such associations only were demonstrable in cohorts consisting of more than one hundred subjects (231). Additionally, alterations in disease activity measures such as DAS28, VASs and HAQ might generally reflect changes that are of importance in the evaluation of rheumatic disease activity, but not to the vasculature. We showed that the DAS28 and patients' and physician's assessment of global disease activity on VASs, but not CRP, ESR, AIX or aPWV improved significantly between infliximab infusions in patients on long-term infliximab therapy (paper 1). CRP evidently reflects the total atherosclerotic risk (232). However, recent results from studies examining the relationship between different CRP genotypes, which influence natural variations in circulating CRP levels, and CVD risk argue against a causal association between CRP and CVD (233). Similar results have also been reported in studies exploring the influence of CRP genotypes on aortic stiffness. Schumacher et al have recently demonstrated that although circulating CRP levels and aPWV were significantly correlated, CRP genotypes and aPWV were not (227). Thus, the observed associations between vascular pathology, CVD and CRP might be due to reversed causality or residual confounding, and not fully reflect the vascular inflammatory processes.

Calprotectin has for several years been an accepted biomarker reflecting disease activity in inflammatory arthropathies (chapter 1.6). During the later years, the heterodimer has also been increasingly associated with CVD. Previous studies in apparently healthy postmenopausal women and in patients presenting with acute coronary syndromes, have demonstrated that elevated plasma calprotectin levels predicted cardiovascular events independently of traditional cardiovascular risk factors (234;235). Furthermore, the protein complex has been shown to be a marker of unstable plaques and an early and sensitive marker of myocardial necrosis in the setting of chest pain (236;237). Croce et al have recently reported that calprotectin promotes vascular inflammation by recruitment of neutrophils and increases neointimal thickening and smooth muscle proliferation in a MRP-14 knockout mice model (238). Many of calprotectin's pro-inflammatory effects are probably mediated by the interaction with the TLR-4, which was recently identified as a receptor for calprotectin, and RAGE (chapter 1.6) (239;240). In the normal artery wall, TLR-4 is expressed by endothelial

cells, whereas in the atherosclerotic artery wall, also macrophages display TLRs. RAGE is expressed by macrophages, endothelial cells and smooth muscle cells. The main intracellular signaling pathway for both TLR-4 and RAGE includes activation of NF- κ B, which leads to enhanced expression of pro-inflammatory cytokines (127). Thus, calprotectin might have a direct role in vascular pathology. We demonstrated that calprotectin was significantly associated with aPWV, but not to CIMT (paper 3). These findings may indicate that calprotectin is one determinant of aortic stiffening in inflammatory arthropathies, and a more sensitive measure of the vascular inflammatory processes than other inflammatory markers included in our studies. However, the association between calprotectin and aPWV does not prove causality. Although experimental results have indicated a role for calprotectin in inflammatory driven intima media thickening (238), data regarding the relation between circulating calprotectin and CIMT is limited (134). The lack of an association between calprotectin and CIMT in our population may reflect the relatively limited number of study participants (231), or the patients' low CIMT levels. Thus, further studies are needed to clarify calprotectin's role in arterial stiffening and intima media thickening.

6 Conclusions and clinical implications

6.1 Conclusions

1. Infliximab infusions did not alter aPWV or AIx in patients with inflammatory arthropathies who were on long-term infliximab therapy (paper 1).
2. Anti-TNF- α therapy improved aPWV after three months, and the beneficial treatment effect was maintained throughout the one-year follow-up period in patients with inflammatory arthropathies, but did not influence AIx (paper 2 and 3).
3. One year of anti-TNF- α therapy reduced CIMT progression in patients with inflammatory arthropathies (paper 3).
4. Calprotectin was longitudinally associated with aPWV after initiation of anti-TNF- α therapy in patients with inflammatory arthropathies. There were no significant correlations between changes in aPWV, CRP and ESR. CIMT and AIx were not associated with inflammatory markers, neither at baseline nor longitudinally (paper 2 and 3).
5. Disease activity assessed by VASs, HAQ and DAS 28 was not associated with aPWV, AIx and CIMT (paper 1, 2 and 3).
6. Treatment with TNF- α antagonists increased plasma L-arginine and the L-arginine/ADMA ratio but did not affect ADMA in patients with inflammatory arthropathies. Plasma ADMA levels and the L-arginine/ADMA ratio were cross-sectionally associated with aPWV, and the L-arginine/ADMA ratio was continuously associated with aPWV after initiation of anti-TNF- α therapy (paper 4).

6.2 Clinical implications

We demonstrated in this thesis a beneficial effect of anti-inflammatory therapy targeting the pro-inflammatory cytokine TNF- α on vascular function and structure in patients with inflammatory arthropathies. Patients with RA, AS and PsA are at an increased risk for CVD largely attributable to a chronic systemic inflammation. Our results indicate that the pro-inflammatory protein calprotectin is associated with arterial stiffness, and might be one mediator of vascular pathology in patients with inflammatory arthropathies. Primary and secondary CVD prevention in patients with RA, AS and PsA should follow the current

national cardiovascular prevention guidelines (241). However, it also seems important to aim at controlling the inflammatory activity in these patients with regard to their CVD risk. Our results suggest that arterial stiffening in inflammatory arthropathies might be reversible, and that the beneficial effect of anti-inflammatory therapy might occur shortly after initiation of treatment. The favorable effect of TNF- α antagonists on CIMT progression shown in this thesis might imply a possible reduction in cardiovascular events due to anti-inflammatory therapy in RA, AS and PsA. However, results regarding long-term cardiovascular outcomes after anti-inflammatory treatment in inflammatory arthropathies are limited, and further research within this field is needed to improve cardiovascular prevention strategies in these patients.

The relevance of our findings in patients with CVD but without a chronic inflammatory disease is uncertain. To date, anti-inflammatory treatment strategies in CVD have not been very successful, and anti-TNF- α therapy may worsen heart failure. However, our results support the view that inflammatory processes are important in vascular pathology.

7 References

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8 Papers

The L-arginine/asymmetric dimethylarginine ratio is improved by anti-Tumor Necrosis Factor- α therapy in inflammatory arthropathies.
Associations with aortic stiffness.

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Abstract

Background: Anti-Tumor Necrosis Factor (TNF)- α therapy seems to improve vascular pathology in patients with inflammatory arthropathies such as rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis. Asymmetric dimethylarginine (ADMA) competes with L-arginine as a substrate for nitric oxide synthase (NOS) and the L-arginine/ADMA ratio is important for modulation of the NOS activity. We examined the effect of TNF- α antagonists on circulatory ADMA and L-arginine/ADMA, and associations between ADMA, L-arginine/ADMA, aortic stiffness and carotid intima media thickness (CIMT) in patients with inflammatory arthropathies.

Methods: Fifty-five patients with a clinical indication for anti-TNF- α therapy were included. 36 patients started with anti-TNF- α therapy and were compared with a non-treated group of 19 patients. Plasma ADMA, L-arginine and aortic stiffness (aortic pulse wave velocity, aPWV) were assessed at baseline, 3 and 12 months. CIMT was examined at baseline and 12 months.

Results: Anti-TNF- α therapy for 12 months increased the L-arginine/ADMA ratio (mean [SD]) in the treatment group compared to the control group (9 [30] vs. -7 [22], $P=0.03$), but did not affect ADMA (0.01 [0.09] $\mu\text{mol/L}$ vs. 0.00 [0.09] $\mu\text{mol/L}$, $P=0.88$). Baseline aPWV was associated with ADMA ($P=0.02$) and L-arginine/ADMA ($P=0.02$) in multiple regression analyses, and the association between the L-arginine/ADMA ratio and aPWV was maintained after initiation of anti-TNF- α therapy ($P=0.03$). ADMA and L-arginine/ADMA were not correlated with CIMT.

Conclusion: Anti-TNF- α therapy improved the L-arginine/ADMA ratio in patients with inflammatory arthropathies. ADMA and the L-arginine/ADMA ratio were associated with aPWV, and may have a mechanistic role in the aortic stiffening observed in these patients.

Key words: ADMA, anti-TNF- α , rheumatoid arthritis, arterial stiffness, carotid intima media thickness.

Introduction

Chronic inflammatory arthropathies such as rheumatoid arthritis (RA), ankylosing spondylitis (AS) and psoriatic arthritis (PsA) are associated with premature aortic stiffening and accelerated atherosclerosis (1;2). Previous results indicate that inflammatory mechanisms are involved in the vascular pathology in these patients. The pro-inflammatory cytokine Tumor Necrosis Factor- α (TNF- α) is central in inflammatory initiation and amplification in both inflammatory arthropathies and atherosclerosis. We have recently reported that treatment with TNF- α antagonists improved aortic stiffness and carotid intima media thickness (CIMT) progression in patients with inflammatory arthropathies (3;4).

Endothelium derived nitric oxide (NO) is an important vasodilator and regulator of vascular homeostasis. NO is produced from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS). Asymmetric dimethylarginine (ADMA) is an endogenous NOS inhibitor, which competes with L-arginine as a substrate for NOS (5). The relationship between ADMA and L-arginine, expressed as the L-arginine/ADMA ratio, is suggested to be important for modulation of NOS activity (6). Circulating ADMA levels have previously been shown to independently predict myocardial infarctions in the general population (7) and cardiovascular events in patients who had undergone percutaneous coronary intervention (8). Recent reports indicate that patients with inflammatory arthropathies have increased plasma levels of ADMA compared to both healthy controls and patients with osteoarthritis (9-14). TNF- α has previously been demonstrated to increase ADMA levels in cell culture studies (15;16). Thus, increased ADMA and/or reduced L-arginine/ADMA ratio may contribute to the vascular pathology seen in inflammatory arthropathies, and anti-TNF- α therapy might be beneficial in this aspect.

The objectives of the present study were to explore the effect of anti-TNF- α therapy on plasma ADMA levels and the L-arginine/ADMA ratio in patients with inflammatory arthropathies, and to examine associations between ADMA, the L-arginine/ADMA ratio and aortic stiffness and CIMT.

Methods

Design and patients

Sixty patients with RA, AS or PsA were recruited from two major rheumatology outpatient clinics in the Oslo area to a prospective, non randomized study as reported in detail previously (3;4). In brief, inclusion criteria were an active inflammatory disease and a clinical indication for anti-TNF- α therapy. Exclusion criteria for the present analyses were uncontrolled arterial hypertension (systolic pressure ≥ 140 mmHg and/or diastolic pressure ≥ 90 mmHg), reduced kidney function (serum creatinine > 133 mmol/L for men and > 124 mmol/L for women) and diabetes mellitus (fasting plasma glucose ≥ 7.0 mmol/L or current use of anti-diabetic medication). Patients with changes in anti-hypertensive or lipid-lowering medication, or patients who initiated treatment with other biological disease-modifying anti-rheumatic therapy such as treatment targeting B-cells (rituximab), T-cells (abatacept), IL-1 (anakinra) or IL -6 (tocilizumab) during the study period would be excluded.

The present study included vascular measurements and fasting blood samples from the baseline (immediately before a possible start with a TNF- α antagonist), three and 12 months examinations. CIMT was measured at baseline and at 12 months. Five patients ended therapy during the follow-up period due to treatment failure, allergic reactions or suspected cancer, and were excluded from the analyses. Thirty-six patients continued anti-TNF- α treatment for one year (17 with etanercept, 10 with adalimumab, and 9 with infliximab), and these patients

were compared to a control group of 19 patients who remained without anti-TNF- α treatment during the total follow-up period. Reasons to postpone therapy initiation were positive Mantoux' test, planned operations, fear of side effects, or co- morbidities. Approval was obtained from the regional research ethics committee, and written informed consent was obtained from each participant. The study was performed according to the Helsinki-declaration.

Vascular measurements

Patients were examined in a quiet, temperature-controlled room after an overnight fast. All examinations were performed with the patients in a supine position after a minimum of 10 minutes relaxation. Systolic and diastolic brachial blood pressures were measured with an appropriately sized cuff using a validated automated device (Omron HEM-757, Kyoto, Japan).

Aortic stiffness, central hemodynamic measures and the augmentation index (AIx) were assessed with the Sphygmocor device version 7.1 (AtCor Medical, Sydney, Australia) and a validated tonometer (SPC-301; Millar instruments, Houston, USA) as previously reported (4). Aortic stiffness was assessed as carotid-femoral pulse wave velocity, which is considered to give an estimate of aortic pulse wave velocity (aPWV). The wave travel distance was obtained by subtracting the distance from the carotid location to the sternal notch from the distance between the sternal notch and the femoral site of recording. Mean arterial pressure (MAP) was determined from the pressure waveforms obtained at the radial artery calibrated with brachial blood pressures.

CIMT was measured in the common carotid artery (CCA) using the multiarray echotracking system Art.Lab (Esaote, Maastricht, the Netherlands) equipped with a 10-5 MHz linear array transducer as previously reported (3). The system utilizes rough radio frequency data to automatically register IMT of the CCA for each subsequent cardiac cycle over a period of 6 seconds (17). Measurements were performed in the far wall of approximately 2 cm long segments in the distal CCA close to the carotid bulb on both sides. CIMT measurements in two patients were excluded from analyses because of insufficient quality.

All vascular and blood pressure measurements were made in triplicate by the same examiner (KA), and their mean was used in the analyses. The repeatability for the vascular measurements are previously reported (3). The examiner did not participate in the treatment of the rheumatic disorder, and information of the patients' treatment was disclosed after completion of data collection and analyses.

Laboratory measurements

Erythrocyte sedimentation rate (ESR), total cholesterol (TC), low density lipoprotein cholesterol (LDL), high density lipoprotein cholesterol (HDL), triglycerides (TG), creatinine and C-reactive protein (CRP) were determined by routine methodology at the time of examinations. EDTA plasma samples were stored at -80°C until analyzed for L-arginine and ADMA, determined by high performance liquid chromatography (HPLC) and precolumn derivatization with o-phthalaldehyde (OPA) (Sigma Chemicals Co, St.Louis, MO) as previously described (18). The inter-assay CV were $< 5\%$ for both.

Statistical analyses

Continuous data are presented as mean with standard deviation or as geometric mean with 95% confidence interval in skewed variables. Comparison of continuous data was done with Student's independent sample test, using log-transformed values in the case of skewed variables. Categorical variables are expressed as numbers and were compared with Pearson's χ^2 test. Baseline associations with aPWV and CIMT as dependent variables were examined with multiple linear regression analyses. Associations between the vascular parameters and ADMA or L-arginine/ADMA adjusted for anti-TNF- α therapy over time were explored using multivariable mixed linear regression models, which included the interaction between time and anti-TNF- α therapy. We used an unstructured covariance structure in the mixed model analyses. Mixed model repeated measures analysis is a linear regression analysis that controls for multiple testing of the same patient by modeling the covariance between the repeated measurements of each individual as a clustered random effect. Variables that were associated (P -value < 0.25) with the vascular measurements in bivariate analyses, or variables known to influence these measurements, were entered into the linear regression and mixed models and subsequently removed in a step-down manner according to levels of significance. Because ADMA is correlated with kidney function and body weight, all regression analyses were adjusted for creatinine and body mass index (BMI). The models were examined for relevant interactions and confounding in a standard manner. P -values ≤ 0.05 were considered significant. Statistical analyses were performed with SPSS, version 18.0 (SPSS Inc., Chicago, USA) and R (R Development Core Team, 2008. R Foundation for Statistical Computing, Vienna, Austria).

Results

Patients' demographic, baseline biochemical and hemodynamic parameters did not differ between the anti-TNF- α group and the control group (Table 1). Geometric means were preferred to median values due to the use of non-transformed values in the regression analyses.

Changes in L-arginine, ADMA and L-arginine/ADMA in the two patients groups from baseline to 3 and 12 months, respectively, are presented in Table 2. These values were normally distributed, and are therefore presented as mean with standard deviation. L-arginine and L-arginine/ADMA levels were significantly improved in the group receiving anti-TNF- α therapy compared to the control group different in the two patients groups both at the 3 and 12 months visits. ADMA plasma levels did not change in any of the two groups during the follow-up period.

The final multiple linear regression analyses with baseline aPWV as dependent variable are presented in Table 3 a) and b). Baseline aPWV was significantly associated with both ADMA ($P=0.02$) and the L-arginine/ADMA ratio ($P=0.02$). L-arginine was not associated with baseline aPWV. Separate models with ADMA and L-arginine/ADMA as independent variables are presented due to the high co-linearity between these variables. Non-transformed or log-transformed values of ADMA and L-arginine/ADMA gave similar results, and the results from the analyses with the non-transformed values are presented. The validity of the models was confirmed by the normal distribution of the residuals. Neither L-arginine, ADMA nor the L-arginine/ADMA ratio was associated with A1x, central blood pressures or CIMT (data not shown).

In the multivariable mixed regression analyses, which included the variable anti-TNF- α therapy over time, the L-arginine/ADMA ratio was associated with aPWV (Table 4), whereas L-arginine and ADMA were not (data not shown). L-arginine, ADMA and L-arginine/ADMA

were neither associated with AIx, central blood pressures nor CIMT (data not shown). Log-transformed values of ADMA, L-arginine or L-arginine/ADMA gave similar results.

The diagnose of inflammatory arthropathy, comorbidities, HR, use of anti rheumatic therapy besides TNF- α antagonists, or biochemical variables such as CRP, ESR, creatinine, TC, LDL, HDL and TG were not associated with aPWV in any of the regression models (data not shown). All regression models were adjusted for creatinine and BMI.

Discussion

The present study examined the effect of treatment with TNF- α antagonists on plasma ADMA, L-arginine and L-arginine/ADMA levels in patients with inflammatory arthropathies. The patients receiving anti-TNF- α therapy showed improved L-arginine/ADMA ratio compared to the patients in the control group, but anti-TNF- α therapy did not affect ADMA levels. Plasma ADMA levels and the L-arginine/ADMA ratio were associated with aPWV, and the L-arginine/ADMA ratio was longitudinally related to aPWV after initiation of anti-TNF- α therapy.

Vascular pathology is considered to be associated with the inflammatory activity in patients with RA, AS and PsA (1;2). Unfavourable changes in the L-arginine/ADMA ratio driven by inflammatory processes might be one part of the explanation for this association.

Inflammatory markers such as CRP and IL-6 have been demonstrated to correlate with reduced circulatory L-arginine and L-arginine/ADMA ratio and increased ADMA in several patient populations (10;11;13;14;19) as well as in the general population (14;19;20).

Furthermore, Antoniadou et al have recently reported that systemic low grade inflammation after *Salmonella typhi* vaccination increased serum ADMA levels and concordantly reduced

FMD in healthy individuals (14). Several previous studies have shown a beneficial effect of anti-inflammatory treatment on vascular pathology in patients with inflammatory arthropathies (1;3;4;21). However, the effects of such therapy on plasma ADMA levels and the L-arginine/ADMA ratio have to our knowledge not previously been examined in longitudinal studies. The observed favourable effect of anti-TNF- α therapy on the L-arginine/ADMA ratio in the present study was related to a change in L-arginine levels but not in ADMA levels. Inflammatory processes have been suggested to decrease L-arginine levels and consequently the L-arginine/ADMA ratio by increasing the activity of arginase, an enzyme that converts L-arginine into L-ornithine, and by reduction of L-arginine transport into endothelial cells by the γ^+ -transporter system (22). Somewhat surprising, we could not find any effect of anti-TNF- α therapy on ADMA levels in our population. TNF- α has previously been demonstrated to increase the ADMA concentration in human endothelial cells in vitro by reducing the activity of the enzyme dimethylarginine dimethyl-aminohydrolase (DDAH) (16). DDAH is the main catabolic pathway for ADMA, and the activity of DDAH strongly affects plasma ADMA levels (23). However, two previous cross sectional studies in patients with AS have reported that serum ADMA levels in patients exposed to a TNF- α antagonist were equal to the levels in conventionally treated patients (9;10), and previous intervention studies with statins and antagonists of the renin-angiotensin system have revealed inconsistent results with regard to the effect of medical intervention on circulatory ADMA levels (24). These results may indicate that a reduction in ADMA is difficult to demonstrate. Furthermore, the baseline ADMA levels in our population were compared to previous reports in the normal range, whereas the L-arginine/ADMA ratio was in the lower range (Table 1) (6). Thus, the potential for a reduction in ADMA might have been limited in our population.

AIx and aPWV are both influenced by endothelial function, and have previously been demonstrated to increase after administration of ADMA or another endogenous NOS

inhibitor, N-monomethylarginine (L-NMMA) (25-27). ADMA inhibition of NO synthesis may reduce the NO mediated smooth muscle relaxation and thus the arteries' capacity of vasodilatation (28). In our population, ADMA and the L-arginine/ADMA ratio were independently associated with baseline aPWV independent of related covariates (Table 3). These findings correspond with a previous report of an inverse correlation between ADMA levels and coronary flow reserve in patients with RA (12). On the other hand, Kemeny-Beke et al have recently reported that FMD in the brachial artery and serum ADMA levels in patients with AS were not correlated (9). However, in the latter study, approximately half of the patients were on treatment with a TNF- α antagonist. Anti-TNF- α therapy has previously been demonstrated to improve FMD (1;21), and treatment with TNF- α antagonists may have influenced a possible correlation between FMD and ADMA. We have previously reported that improvement in aortic stiffness after initiation of treatment with TNF- α antagonist predominantly occurred during the first three months of therapy (3). In the present study, the main change in the L-arginine/ADMA ratio *also* took place in the same period (Table 2), and the L-arginine/ADMA ratio was associated with aPWV in the multivariable analysis which included the interaction variable anti-TNF- α therapy x time (Table 4). Thus, improvement in the L-arginine/ADMA ratio may have contributed to the observed improvement in aPWV during anti-TNF- α therapy. We could not demonstrate any correlation between central pressures or AIx and ADMA or the L-arginine/ADMA ratio. These negative findings may be due to lack of power, but previous results have indicated that aortic stiffness might be a better measure of arterial stiffening than AIx in patients with inflammatory arthropathies (1;3;4). This difference in sensitivity might be due to the peripheral vasodilatation associated with systemic inflammation, which would counteract the effect of the inflammation induced aortic stiffening on pulse wave reflections and the AIx.

Previous studies indicate that circulating ADMA levels are associated with CIMT (20;29) and predict CIMT progression (29) in the general population, although a large cross sectional study in a Framingham heart study offspring cohort found significant association only to internal carotid artery or carotid bulb IMT and not to CIMT (30). Data regarding the correlation between ADMA and CIMT in patients with inflammatory arthropathies are to date inconsistent (9;11;12), and we did not observe any association between CIMT and ADMA, L-arginine or the L-arginine/ADMA ratio. However, the patients in the present study had CIMT values within the normal range (Table 1), and correlations between ADMA, L-arginine and CIMT may be easier to judge in higher age groups with more pronounced atherosclerosis or in larger patients groups.

The present study has some limitations. The patients were not randomized to treatment or placebo due to ethical reasons. The non-randomized design could have introduced potential bias. However, the treatment and control groups were recruited from the same clinical setting and the two patient groups did not differ with regard to demographic characteristics or baseline inflammatory and hemodynamic measures. In our previous reports from the same patient population, repeated analyses after matching the patients by age, sex and MAP did not change the results (4). Furthermore, the associations between aPWV and ADMA and the L-arginine/ADMA ratio in the present study are not proven to be causal, and need further exploration in future studies.

In conclusion, initiation of anti-TNF- α therapy improved the plasma L-arginine/ADMA ratio, but did not influence plasma ADMA levels in patients with inflammatory arthropathies. Plasma ADMA levels and the L-arginine/ADMA ratio were associated with baseline aPWV, and the L-arginine/ADMA ratio was furthermore longitudinally associated with aPWV after initiation of anti-TNF- α therapy. These findings indicate that ADMA and L-arginine/ADMA

may have a mechanistic role in the aortic stiffening seen in patients with inflammatory arthropathies.

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Table 1. Patients' demographic and baseline biochemical and hemodynamic parameters

	Anti-TNF- α (n=36)	Control (n=19)	P
<i>Demographic</i>			
Patient age, yrs	47.2 (11.6)	51.9 (14.5)	0.16
Females / males, n	14 / 22	8 / 11	0.82
Disease duration, yrs	10.1 (8.8)	11.9 (11.3)	0.50
RA / AS / PsA, n	15 / 12 / 9	10 / 7 / 2	0.43
BMI, kg/m ²	26.0 (6.7)	26.0 (3.1)	0.94
<i>Biochemical</i>			
L-arginine, μ mol/L	78 [74, 82]	78 [71, 86]	0.86
ADMA, μ mol/L	0.59 [0.54, 0.65]	0.58 [0.50, 0.68]	0.83
L-arginine/ADMA, ratio	132 [119, 145]	136 [117, 156]	0.75
Creatinine, μ mol/L	67.9 [63.2, 72.5]	72.2 [66.0, 79.0]	0.17
CRP, mg/ L	5.6 [3.2, 8.8]	5.4 [3.0, 9.6]	0.88
ESR, mm/h	13.0 [9.9, 17.2]	12.7 [7.1-22]	0.95
<i>Hemodynamic</i>			
aPWV, m/s	7.48 (1.60)	7.50 (1.00)	0.95
CIMT, mm*	0.560 (0.112)	0.563 (0.110)	0.95
Brachial SBP, mm Hg	131 (19)	131 (22)	0.99
Brachial DBP, mm Hg	79 (11)	78 (11)	0.69
Central SBP, mm Hg	120 (19)	123 (21)	0.68
Central PP, mm Hg	40 (11)	44 (13)	0.22
MAP, mm Hg	98 (14)	97 (14)	0.88
HR, bpm	64 (9)	62 (10)	0.46
AIx, %	20.0 (12.1)	22.9 (10.8)	0.38

SBP indicates systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; HR, heart rate; *n=34 in the Anti-TNF- α group. Values are represented as mean (SD) or numbers, except for ADMA, L-arginine, L-arginine/ADMA, creatinine, CRP and ESR which were skewed and are represented as geometric mean [95% confidence interval].

Table 2. Changes from baseline in L-arginine, ADMA and the L-arginine/ADMA ratio

	3 months			12 months		
	Anti-TNF- α (n=36)	Control (n=19)	P	Anti-TNF- α (n=36)	Control (n=19)	P
L-arginine, $\mu\text{mol/L}$	6 (17)	-3 (10)	0.03	6 (10)	-1 (14)	0.03
ADMA, $\mu\text{mol/L}$	0.01 (0.10)	0.02 (0.07)	0.72	0.01 (0.09)	0.00 (0.09)	0.88
L-arginine/ADMA	10 (35)	-13 (20)	0.01	9 (30)	-7 (22)	0.03

Values are given as mean (SD). P values refer to between group comparisons of changes after

3 and 12 months, respectively.

Table 3 a) and b). Multiple linear regression analyses for baseline associations with aPWV

a)	Regression Coefficient (95% CI)	P
Male sex	0.31 (-0.26, 0.87)	0.41
Age, years	0.07 (0.04, 0.09)	<0.001
MAP, mmHg	0.03 (0.01, 0.06)	0.003
ADMA, $\mu\text{mol/L}$	1.70 (0.27, 3.35)	0.02
b)	Regression Coefficient (95% CI)	P
Male Sex	0.32 (-0.16, 1.03)	0.15
Age, years	0.07 (0.04, 0.09)	<0.001
MAP, mmHg	0.03 (0.08, 0.055)	0.01
L-arginine/ADMA	-0.01 (-0.02, -0.00)	0.02
CI indicates confidence interval.		

Table 4. Multivariable mixed model with aPWV as dependent variable (n=55, 3 visits)

	Regression Coefficient (95% CI)	P
Male sex	0.17 (-0.39, 0.72)	0.55
Age, years	0.07 (0.05, 0.09)	<0.001
Anti-TNF- α therapy	0.42 (-0.15, 1.00)	0.15
Time	-0.03 (-0.05, -0.01)	0.004
Anti-TNF- α therapy \times Time	-0.04 (-0.07, -0.01)	0.02
MAP, mmHg	0.02 (0.01, 0.03)	<0.001
L-arginine/ADMA	-0.004 (-0.007, -0.000)	0.03

CI indicates confidence interval; Anti-TNF- α therapy \times Time, treatment (yes/no) multiplied with months after baseline.